Q fever in Puducherry: Diagnostic utility of Nested PCR, Real-Time PCR and Loop Mediated Isothermal Amplification for detection of *Coxiella burnetii* DNA in antibody positive and negative blood samples

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1. INTRODUCTION

Query fever (QF) is an emerging infectious zoonotic disease which occurs worldwide with exception of New Zealand (Hilbink et al., 1993). It is caused by a gram negative obligate intercellular bacterium *Coxiella burnetii.* Q fever remains under-diagnosed or underestimated. *C.burnetii* is a potential agent of bioterrorism. It is classified under category 'B' pathogen and it requires Bio-safety level III containments facilities (Anderson et al., 2013). Q fever is listed as one of the top 13 zoonoses in the world, based on human as well as livestock impact and severity of the disease (Grace et al., 2012). It is a highly heat resistant spore-like form and the transmission occurs through ingestion of unpasteurized milk/dairy products or inhalation of aborted products from the domestic animals like sheep, goat, cattle and buffaloes (O.I.E., 2013). *C. burnetii* can survive at 1 to 6 °C for six weeks and remains infectious in donated blood products (Kersh et al., 2013). QF is primarily an occupational hazard to farmers, veterinarians, butchers and slaughterhouse workers who are in regular contact with domestic livestock (Anderson et al., 2013).

C. burnetii exists in two phases: Phase I and Phase II, with phase variations from smooth to rough, which is similar to some members of Enterobacteriaecae (Maurin & Raoult, 1999). Phase I is virulent and mostly isolated from naturally infected animals or humans and Phase II is a less infectious, attenuated phase and the changes may occur due to repeated in-vivo or in-vitro serial passages by animal inoculation, yolk sac or tissue cultures (O.I.E, 2013).

QF in humans can manifest as acute, chronic or subclinical form and there is no specific genotype associated with above infections (Maurin & Raoult, 1999; O.I.E, 2013). Clinical symptoms like Fever, Chills, Vomiting, abdominal pain and pneumonia are the typical manifestation of acute Q fever. Endocarditis, granulomatous hepatitis or chronic fatigue syndrome are common manifestation of chronic Q fever (Anderson et al., 2013).

Isolation is restricted only to the reference laboratories (O.I.E., 2013). The serological tests include the older tests like Capillary agglutination test (CAT) (Luoto, 1953), Microagglutination test (MAT) (Babudieri., 1958; Shukla CL, Negi., 1978) and Complement fixation test (CFT) (Fiset et al., 1969). Newer serological tests are Enzyme Linked Immunosorbent Assay (ELISA) (Jager et al., 2011; Herrmann's et al., 2013; Stephen et al., 2017), Indirect Immunofluorescence Assay (IFA) (Jager et al., 2011; Herrmann's et al., 2013; Stephen et al., 2017; Pradeep et al., 2017) and Immunoperoxidase tests (Brouqui et al., 1994) for detection of *C. burnetii* antibodies. Early detection of Q fever is based on molecular methods within 10 to 21 days of febrile illness and at later stages it may not be possible to detect *C. burnetii* DNA when antibodies appear (Schneeberger et al., 2010; Jager et al., 2011).

During the last two decades, Q fever with different presentations like endocarditis (Balakrishnan et al 2008), febrile illness (Stephen et al 2017; Kumar et al., 2017; Pradeep et al., 2017), neonatal sepsis (Arthi et al., 2013) atypical pneumonia (Gangoliya et al., 2016) and abortion in pregnant women (Vaidya et al., 2008) have appeared in Indian literature.

An early diagnosis of *C. burnetii* DNA was detected by PCR using different genes like Insertion sequence (IS), Outer membrane protein (OMP), Isocitrate dehydrogenase (ICD), Superoxide dismutase (SOD) (de Bruin et al., 2013) and DNA Gyrase (Pradeep et al., 2017).

In the present study, we compared the most sensitive and specific gene IS1111-repetitive element, with multicopies and highly conserved with another specific gene, COM1, a single copy number present in the genome (de Bruin et al., 2013). In this study, archived samples which were either positive or negative for *Coxiella burnetii* Ph II IgM ELISA were subjected to the Indirect Immunofluorescent antibody test (IFA). These samples were also subjected to three molecular diagnostic tests namely Nested PCR, Real Time PCR and Loop Mediated Isothermal Amplification (LAMP). LAMP is a new molecular diagnostic tool, which is a simple, easier to

perform and does not require sophisticated equipments. LAMP is highly sensitive and specific, with both outer and inner primers designed for the detection of specific DNA in human clinical samples (Notomi et al., 2000). A limited number of reports on QF LAMP are available in world database (Pan et al., 2013; Raele et al., 2015; Chen and Ching, 2017). Acute febrile patients, animal handlers and healthy controls (voluntary blood donors) were included in this study.

2. AIMS AND OBJECTIVES

Primary Objectives:

1. Confirmation of archived and anonymised blood samples which were either positive or negative for *C. burnetii* Ph II IgM antibodies in ELISA by the 'gold standard' IFA IgM/IgG (Ph I & Ph II).
2. Comparison of IFA positive/negative samples with Nested PCR (N-PCR), Real Time PCR (qPCR) and LAMP targeting the most sensitive and specific IS1111 gene and COM1 gene
3. Compare serological and molecular diagnostic tests with clinical and laboratory parameters.
4. To find out the practical utility and affordability of serological and molecular diagnostic tests.

Secondary Objective:

1. To validate the commercial qPCR (portable qPCR) (Genesig qPCR kit – Primer Design Pvt. Ltd.) for detection of *Coxiella burnetii* DNA in QF IFA positive/negative samples.

3. BRIEF REVIEW OF LITERATURE

3.1 Q FEVER - GLOBAL PREVALENCE:

QF is a worldwide zoonotic infectious disease and it exists in all continents except New Zealand (Hiblink et al., 1993). Most of the developing countries in the world rely on seropositivity, since culture needs level-III containment facilities. Serodiagnosis is mainly based on Ph II IgG antibodies for probable cases and confirmed by either fourfold increase in titer and/or detection of *C. burnetii* DNA in PCR (Anderson et al., 2013).

In the North American continent *C. burnetii* antibody was detected in 3.1% of healthy adults in USA (Kresh et al., 2013). QF was recorded in South American countries like Trinidad in slaughterhouse workers (4.4%) (Adesiyum et al., 2011) and 3.3% febrile patients in Brazil (Maria-Guia et al., 2012). In French Guiana (Elpelbin et al., 2012) 24.4% prevalence was reported among patients with community acquired pneumonia.

Among European countries, Greece had a low prevalence of 2.9% (Maltezou et al., 2004) and seroprevalence of 6% was reported in Austria (Skerget et al., 2003). Diyarbakir region of Turkey had 6.6% seropositivity among stock breeding staff (Arserion et al., 2011). A higher seropositivity of 20% was observed in Northern Ireland (McCaughey C et al., 2008). Coxiellosis on humans was high in Poland (31.12%) (Szyman´ska-Czerwin´ska et al., 2015) followed by France (24.4%) (Alonso et al., 2015). Espeyo et al., described the QF positivity based on seasonal variations: higher in November – February (32.6%), moderate during March – June (16.00%) and less in July – October (13.00%) with pneumonic forms in the cooler months. Nearly, 904 cases were recorded as acute Q fever in England during the period of 2000-2015 (Halsby et al., 2017). Italy showed a prevalence rate of QF among 10.8% prisoners, 16.5% of prison guards and only 3.2% in the residents (Santoro et al., 2004). Major outbreak of QF

occurred during 2007-2009 and more than 2000 cases of Q fever were reported for the first time in the history of Netherlands (Schneeberger et al., 2010). The diagnostic algorithm was proposed for confirming acute/chronic Q fever for the management of QF outbreak (Jager et al., 2011).

In Asia, Q fever outbreak in Central Israel was reported involving 144 out of the 164 tested individuals comprising mostly school children and some employees (Amitai et al., 2010). Afghanistan had a QF seropositivity of 63.9% (Akbarian et al., 2015). About 35.6% cattle farmers in China were seropositive for QF (Sun et al., 2016). Moderate positivity of 13.8% in North western Iran in acute febrile patients was reported by Esmaeili et al., 2017. About 9.6% of seropositivity for *C. burnetii* was found in Orang Asli peoples of Peninsular Malaysia (Khor et al 2018). Taiwan had 7.2% QF cases among community acquired pneumonia (Lai et al 2014) and Sri Lanka recorded 1.6% positivity in febrile patients (Angelakis et al., 2012)

Among African countries, the highest seropositivity was observed in Tanzania, with 55.5% seroprevalence (Prabhu et al., 2011), whereas a moderate seropositivity of 26.1% was reported in Namibia among voluntary blood donors (Noden et al., 2014), followed by Senegal with 24.5% (Mediannikov et al., 2010) and Kenya had 19.1% positivity among febrile patients (Njeru et al., 2016). However, QF was absent from other regions of African continent like Mali, Morocco, Gabon, or Tunisia (Angelakis et al., 2014).

In the Australian continent, the cases occur throughout Australia, with varying positivity: Northern New South Wales (3.1 per 100,000 per annum), Southern Queensland (6.3 per 100,000 per annum), South Australia (1.1 per 100,000 per annum) and <1.0 per 100,000 per annum in the other states and territories (Eastwood et al., 2018).

3.2. Q FEVER IN INDIA:

3.2.1. Q fever in Human:

The first human case of Q fever was reported based on rising antibody titre in follow-up blood samples (Anderson and Kalra, 1954). After two years, a human case of Q fever was reported from Jammu province in a Medical Officer in the Armed Forces, purely on the basis of clinical as well as radiological findings and later confirmed by patient's serum (Ghosh and Rao, 1956). Veeraraghavan and Sukumaran (1954) conducted an extensive serological survey in the Nilgris and Coimbatore districts of Madras state and found that seven had Q fever antibodies with titres ranging from 1:16 to 1:64. In 1959, Kalra has reviewed the status of rickettsial diseases in India. He observed that there was a lack of information about Q fever in India. For the first time in India, Choudhury et al., 1972 documented that the camels are the source of human QF infection and later they conducted the sero-surveillance on human Q fever. The incidence rate was more in rural areas among pediatric age group. The seroprevalence of human Q fever in Jammu and Kashmir was found to be 20.7% by Joshi et al., (1975). None of the 85 sera collected from Delhi were positive for Q fever (Kalra and Taneja, 1954). Regarding Q fever in man and animals, only two reviews are available in Indian literature so far, which appeared in 1978 and 1980, with detailed report of seropositivity and the tests performed by the researchers (Padbidri and Gupta, 1978; Stephen et al., 1980a). In Rajasthan, *C. burnetii* was isolated from human milk (Prasad et al., 1986). Prevalence of QF using complement fixation test (CFT) varied from 0.4% to 23.5 % and by the use of Capillary agglutination test seropositivity ranged from 2.8% to 40.9% (Pathak and Tanwani, 1971; Randhawa et al., 1972; Ghosh et al., 1976; Kulshreshtha et al., 1977; Shanmugam et al., 1978; Stephen et al., 1980). Rao (2003) reported seroprevalence of human Q fever in Rajasthan employing CAT. Applying IFA, seroprevalence

of 26.19%, 25.68% and 8% was reported in Puducherry (Stephen et al., 2017), Uttar Pradesh (Vaidya et al., 2008) and Tamil Nadu (Balakrishnan et al., 2008) respectively.

In India, PCR as a molecular diagnostic tool for identifying cases of Q fever has been utilized since 2008 only. Targeting IS1111 gene, Q fever has been reported in patients with different clinical manifestations like human abortions (21.62%) (Vaidya et al., 2008), neonatal sepsis (Arthi et al., 2013), atypical pneumonia (Gangoliya et al., 2016), acute febrile illness (4.25%) (Kumar et al., 2017) and pneumonia (Panjwani et al., 2015). Another new target DNA Gyrase (97kDa gyrA gene) could identify only less number of cases (2.78%) among febrile patients with lung involvement (Pradeep et al., 2017). Two molecular studies identified genetical evidence of *C. burnetii* in reproductive products of animals (Das et al., 2013) and febrile patients (Kumar et al., 2017).

3.2.2. Coxiellosis in animals

The first isolation of *C. burnetii* from animal milk samples was reported by Kalra and Taneja. Seroprevalence of coxiellosis in domestic livestock has been reported from every parts of India by either serological or molecular methods. A review on the seroprevalence of QF among different species of animals recorded a varied picture in different animals: 3.3% - 49.7%, 1.92% - 14.3%, 0.97% - 16.2% and 1.06% - 35.7% in sheep, goat, cattle and buffaloes respectively. Other domestic animals like horses had high positivity of 53.8%, followed by dogs (34.62%), mules (22.95%), camels (11.92 – 24.9%), pigs (9.5%), fowls (8.1%) and bullocks (2.85% - 5.33%) by using CFT, CAT, MAT (Choudhury et al., 1971; Randhawa et al., 1972; Joshi et al., 1975; Ghosh et al., 1976; Kulshreshtha et al., 1977; Padbidri and Gupta, 1978; Shukla and Negi, 1978; Stephen et al., 1980) and ELISA (Stephen et al., 2014; Pradeep et al., 2016; Dhaka et al., 2017). Milk of domestic livestock and camels are the major reservoirs of QF infection in Punjab and Rajasthan state during early seventies (Randhawa et al., 1973). A limited number of

scientific reports are now available regarding detection of *C. burnetii* DNA in domestic animals from three states viz., Uttar Pradesh (6.8% - Das et al., 2014); (6.6% - Vaidya et al., 2010), Kerala (0.75% - Dhaka et al., 2017) and Puducherry (4.26% - Pradeep et al., 2017a) employing Trans PCR.

4. MATERIALS AND METHODS

Type of Study: Prospective and investigative laboratory based study utilizing archived and anonymised serum samples

Place of Study:

1. Department of Microbiology, Mahatma Gandhi Medical College & Research Institute (MGMCRI) and

2. Department of Proteomics and Genomics, Centre for Interdisciplinary Research Facility (CIDRF), MGMC & RI Campus, Sri Balaji Vidyapeeth (Deemed University), Puducherry.

Study Period: November, 2015 to March, 2018

Ethics approval:

Approval was obtained from our Institutional Human Ethical committee (IHEC), MGMC & RI. Approval Project No: PG DISSERTATION/2015/10/02, dated 30th November 2015.

Specimen Collection:

Sample size in each group: Based on the 87.5% sensitivity of the antibody tests, sample size (n) was calculated using the formula:

$$\text{“n} = \frac{1.96^2 \text{ (PQ)}}{d^2}$$

Where confidence level at 95% (Standard value of 1.96)

P = 87.5 (Sensitivity of the antibody tests)

Q = 1-P = 12.5

$d^2 = 36$ (Margin of error at 6%)

Antibody positive samples → 150* (*Samples to be tested are 116, which is rounded upto 120 and 30 more samples were included, totaling 150)

Antibody negative samples →120 as control

Blood samples from out-patients/in-patients, who attended our tertiary hospital with febrile illness (n=150) were archived and anonymised. Other groups were: veterinarians/animal attendants (n=24), butchers/ slaughterhouse workers (n=36), farmers (n=15) and voluntary blood donors (n= 45) from various places in Puducherry and neighbouring Tamil Nadu. Informed written consent from these persons was obtained prior to collection of blood.

Serological and molecular tests were performed for detection of *C. burnetii* antibodies and DNA in human blood samples.

Immunofluorescence Assay (IFA):

Commercial QF IFA IgM Ph I+II (QM-120 - *Coxiella burnetii* IgM IFA antibody kit) and IgG Ph I+II (QG-120 - *Coxiella burnetii* IgG IFA antibody, Fuller Laboratories, California, USA), slides were coated with two antigen spots (Ph I+II) for detection of *C. burnetii* antibodies in human sera. The test was carried out strict adherence of manufacturer's instruction. Briefly, patients' serum samples were initially diluted to 1:16 in IgM sample dilution buffer and IgG with phosphate buffered saline (PBS) provided in these kits. Slides were incubated 90 minutes and 30 minutes for IgM and IgG in humidity chamber respectively. After incubation, slides were washed, gently with PBS and briefly immersed in distilled water and allow the slides to dry. Added 10µl of conjugate into the wells and incubated at 30 minutes for both IgM and IgG. Washing will follow the previous step. After the final wash, the slides were dried, mounted with the mounting medium and read with ×400 magnification, at 390 nm using Primo Star iLED

Fluorescent microscope (Carl Zeiss Micro Imaging, GmbH, Gottingen, Germany). According to this kit, significant titres for Ph I+ II IgM and Ph I IgG were ≥1:16 and for Ph II IgG ≥1:256.

DNA Extraction:

About 200 µl buffy coat samples were used for the genomic DNA extractions as per the procedure of DNA Blood Mini Kit (Qiagen, Germany). The purity of the extracted DNA was determined by absorbance (A) using Eppendorf Spectrophotometer. A260/A280 ratios were in the range of 1.7-1.8 for the 270 samples. The samples were aliquoted and stored at 80°C till the completion of the molecular tests.

Molecular Diagnostic Tests:

For the early detection of *C. burnetii* DNA in blood samples, three molecular diagnostic tests were carried out viz., N-PCR, qPCR and LAMP. Lyophilized DNA of *Coxiella burnetii* Nine Mile RSA 493 strain was purchased from Bioscience, Bratislavia and used as a positive control in the molecular tests.

Nested PCR (N-PCR)

For IS1111 target, the primers used were those of Lorenz et al., 1998. The first set of amplification was observed 687bp and second set at 243bp. Regarding COM1 target, the primers were as described by Zhang et al., 1998. The outer primers amplified at 501bp and inner primers at 438bp. The cyclic conditions and reaction set up is similar for both the targets, but the annealing temperature is different for each set of primers. The PCR was carried out using Applied Bios-system, Veriti Dx 96-wells Thermal cycler (Thermo Fisher Scientific, Singapore). The positive bands are visualized by 2% agarose gel electrophoresis stained with Ethidium bromide at GelDoc^{+} Gel Documentation system (Bio-Rad, USA)

In-house Real Time PCR (qPCR)

Detection of *C. burnetii* DNA was attempted by targeting the same two genes, IS1111 and COM1. For IS1111a primers of Schneeberger et al., 2010 and for COM1, primers of de Bruin et al., 2013 were applied, which amplify at 70bp and 133bp respectively. A standard curve was generated using five log series of tenfold dilution of *C. burnetii* DNA control for both the targets. All samples for qPCR were run in duplicate and Human Beta actin (ACTB) was used as the housekeeping gene. The primers were as suggested by Mediannikov et al., 2010. Ct values for ACTB were at 28.0 ±2.0. Cyclic amplification was 45 cycles, with final volume of 20µl reaction set up was similar for both the gene targets and housekeeping gene, but the annealing temperatures was different.

Commercial Portable q16 qPCR

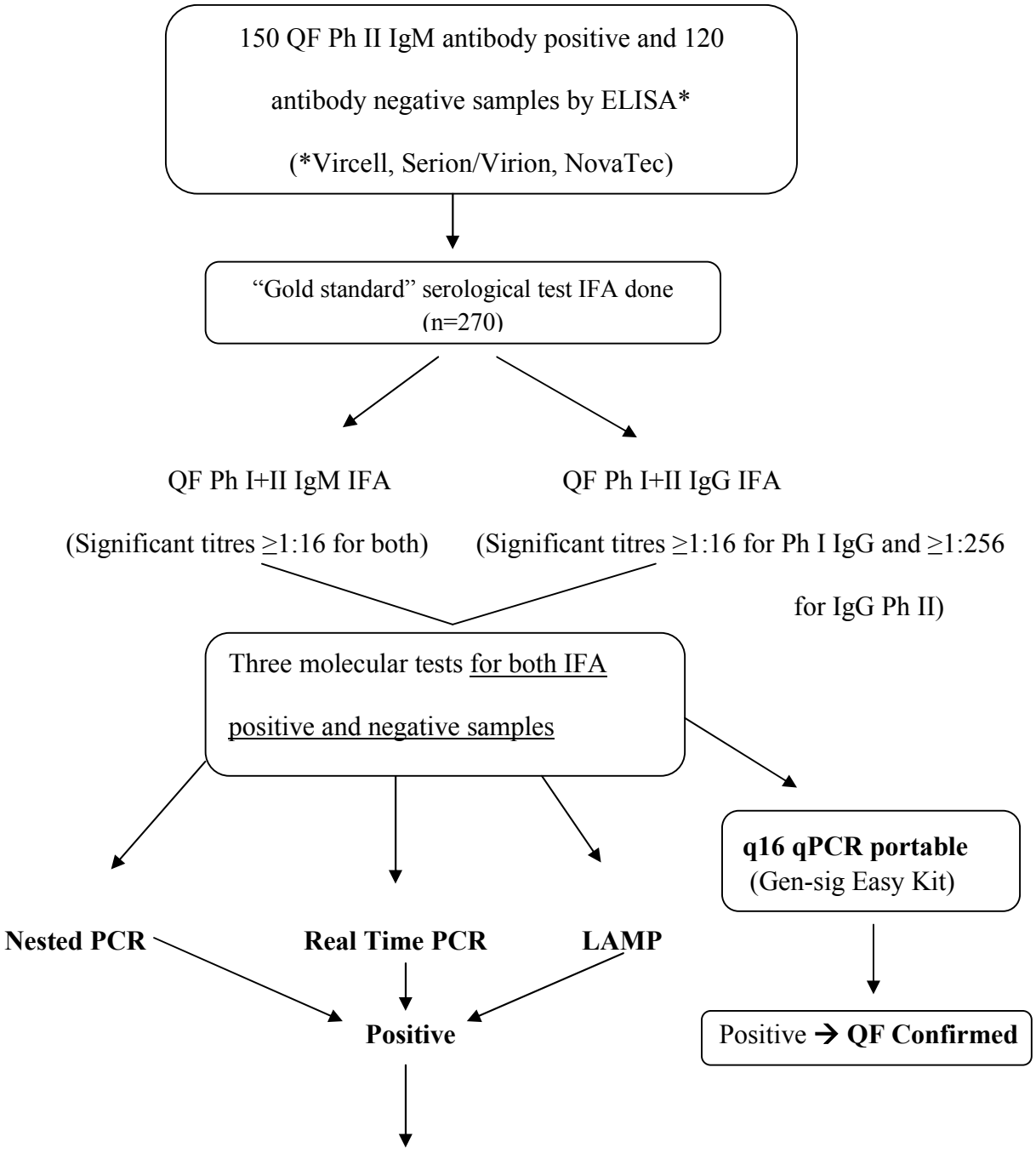
Genesig Easy portable q16 qPCR kit (Primer design Ltd., Southampton, United Kingdom) targets DNA Gyrase subunit A (97kDa gyrA) gene. A reaction mix of 20 µl was prepared with 10 µl of 2 x osaigTM Master mix which contains primers and probes and 10 µl of genomic DNA. According to this kit instruction, amplification was carried out with 45 cycles by combining annealing and extension temperature. Cycle threshold (Ct) values of the positive samples in this commercial qPCR kit were ≤ 45.0.

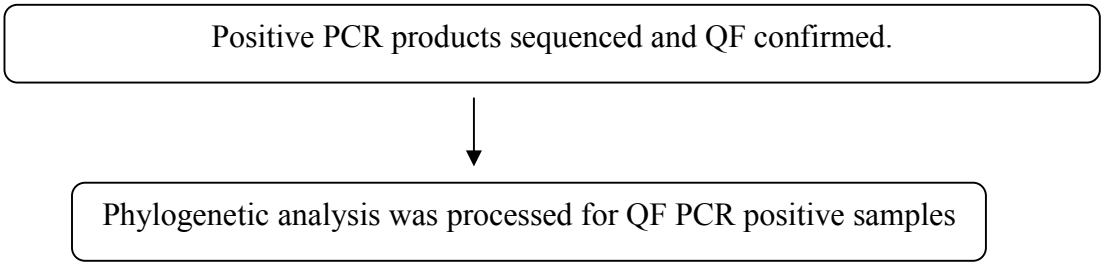
Loop Mediated Isothermal Amplification (LAMP)

Coxiella burnetii strain EVC 282 (GenBank Accession no. KT391019) was selected as the LAMP target for designing four specific primers of IS1111 and COM1 genes as described by Raele et al., 2015, and were synthesized by Sigma Aldrich, Bangalore. LAMP was performed with 25µl of reaction volume comprising of 10X Isothermal amplification buffer II, 5µM outer primers (F3 & B3) and 40µM of inner (FIP & BIP) primers. To this mixture Bst 3.0 DNA polymerase (8000 Units/ml), 5M Betaine, 8mM $MgSO_4$, 1.4mM DNTPs and nuclease free

distilled water. Before amplification, 3mM Hydroxy Naphthol blue was added. After amplification the color was visualized: sky blue for positive and purple for negative reaction. This was also later confirmed by development of ladder like pattern on 2.0% agarose gel electrophoresis stained with Ethidium bromide solution.

The flow chart of the procedure carried out for the molecular tests is as follows:


150 QF Ph II IgM antibody positive and 120 antibody negative samples by ELISA*
(*Vircell, Serion/Virion, NovaTec)
"Gold standard" serological test IFA done (n=270)
QF Ph I+II IgM IFA
(Significant titres ≥1:16 for both)
QF Ph I+II IgG IFA
(Significant titres ≥1:16 for Ph I IgG and ≥1:256 for IgG Ph II)
Three molecular tests for both IFA positive and negative samples
Nested PCR
Real Time PCR
LAMP
q16 qPCR portable
(Gen-sig Easy Kit)
Positive
Positive → QF Confirmed



Gene sequencing:

In the present study, 22 IS1111 and one COM1 gene positive for amplified N-PCR products were purified using QIAquick PCR purification kit (Qiagen, Germany) as per the procedure outlined by the manufacturers. The purified products were subjected for 2.0% agarose gel electrophoresis stained with Ethidium bromide for confirmation. These amplicons were custom sequenced using an Applied Biosystems ABI3730XL.

5. STATISTICAL ANALYSIS

Percentages were calculated for categorical variables. Mean and standard deviations (SD) were calculated with 95% confidence interval for numerical variables. Kappa statistic agreement was performed for three molecular tests. Chi-square tested for numerical variables or Fisher's exact test for small number of samples. P values < 0.05 were considered statistically significant.

6. RESULTS

Among 150 ELISA positive patients, 73 (48.67%) were male, aged from two to 66 years and 77 (51.33%) females, who were from one year to 80 years old. Age wise distribution of QF ELISA positive patients are grouped under: 1-14 years (14.6%), 15-24 (22.67%), 25-34 (20.67%), 35-44 (16.67%), 45-54 (10.0%), 55-64 (10.67%) and more than 65 years (4.67%) and no statistical difference was observed among the above age groups (p=0.912). Among 120 ELISA negative, 109 (90.83%) males with their ages ranging from 13 to 70 years and 11

(9.17%) females in the age group of 15-64 years. Mean age for the patients was 37.51 ±19.30 (32.46 - 42.57). Mean age for animal handlers, 40.93± 12.33 (36.44 - 45.42) and voluntary blood donors 27.35±7.57 (25.14 - 29.56).

The patients' duration of illness was 1–20 days (Mean duration ± SD, 7 ± 4 days). Out of 150 patients, 57 came from rural areas, 55 from urban areas and 38 were residents from Peri-urban dwellers. More positivity was observed in rural patients than the urban and peri-urban population, which is statistically significant (p=0.0012). History of animal contact was observed in 28 patients, 19 in QF positive and nine in QF negative which is statistically significant (p=0.0002).

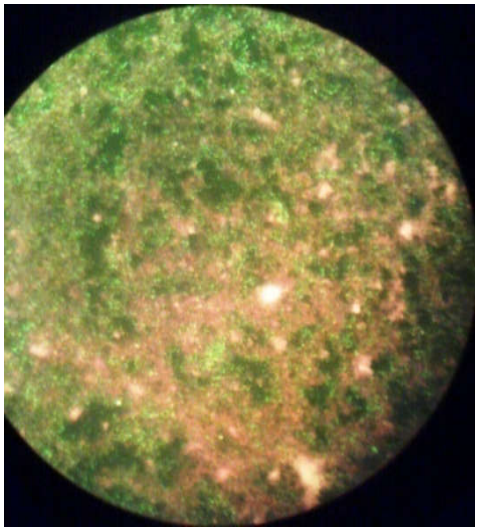
6.1. Immunofluorescence Assay:

Figure– 1 and 2 shows images of QF IFA Ph I/II IgM and Ph I/II IgG along with positive and negative controls. A total of 270 Ph II IgM ELISA samples were archived and subjected for QF IFA IgM and IgG (Ph I/II) with different combination of antibodies. Among 150 Ph II IgM ELISA positive samples 19 were positive in Ph II IgM IFA. Table – 1 shows the details of IFA positivity in different combinations (Ph I IgG/IgM and Ph II IgG/IgM).

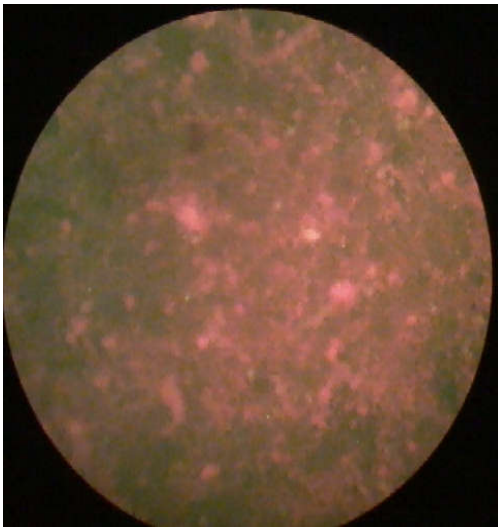
Out of 120 ELISA negative samples, 12 (10.00%) had *C. burnetii* antibodies who were either animal handlers (Veterinarians, Animal attendants, Butchers, Slaughter house workers) or household contacts of domestic livestock. IgG Ph I was seen in three participants, IgG Ph II in eight and IgM Ph II and IgG Ph I + II was positive in one. None of them had antibodies to Ph I IgM. The highest titre was 1:32 for IgM Ph II, whereas it was 1:512 for IgG Ph I and II. All 45 voluntary blood donors were seronegative for QF (Table – 2).

Figure – 1: Images of QF IFA IgM (Ph I & II antibodies)

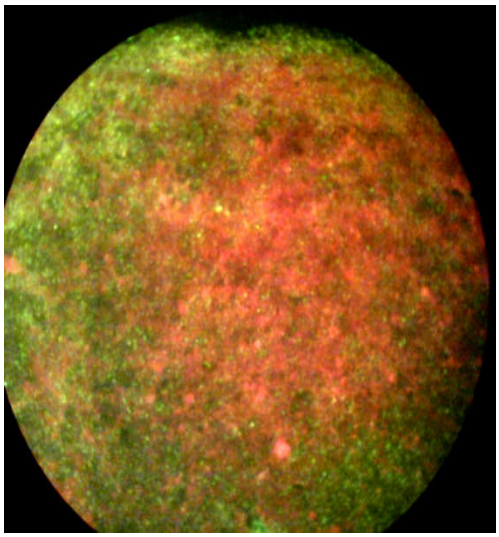
1a. Positive control for IgM (1:32)



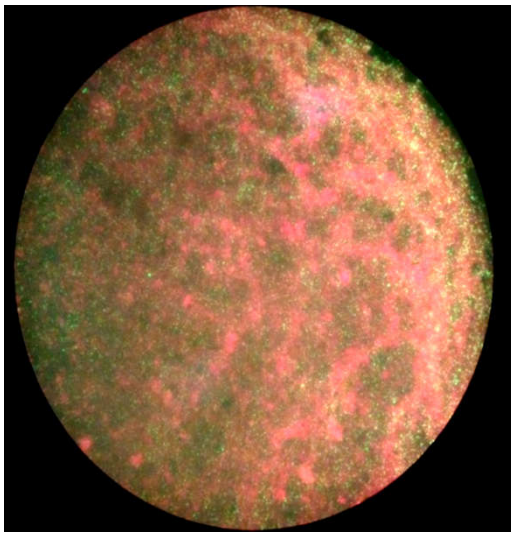
1b. Negative control for IgM (≤1:16)



1c. QF IFA Ph I IgM (1:64)



1d. QF IFA Ph II IgM (1:128)

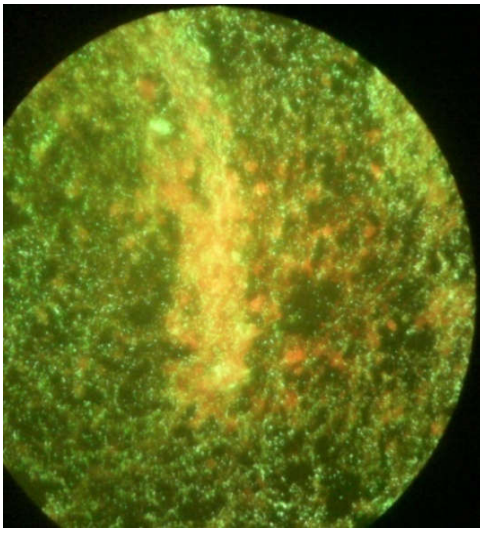


1a & 1b shows Positive control (1:32) and Negative control (1:16) for IgM;

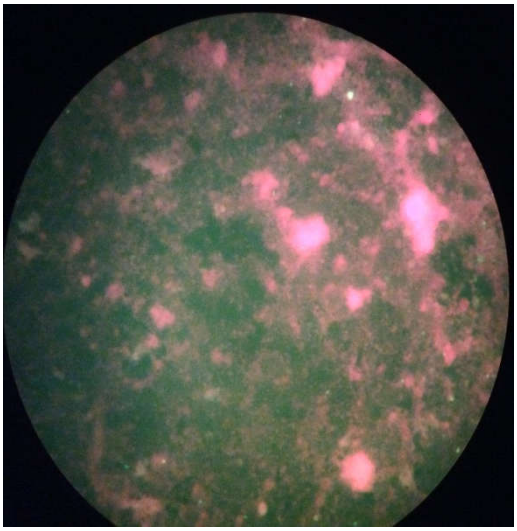
1c & 1d shows test positive for dilution 1:64 for Ph I IgM and 1:128 for Ph II IgM

Figure – 2: Results of QF IFA IgM (Ph I & II antibodies

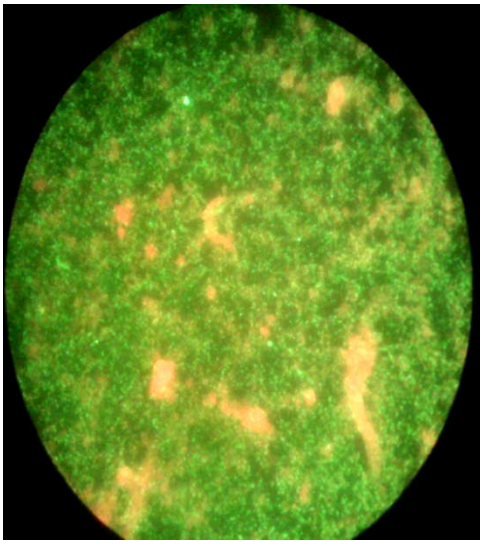
2a. Positive control for IgG (1:32)



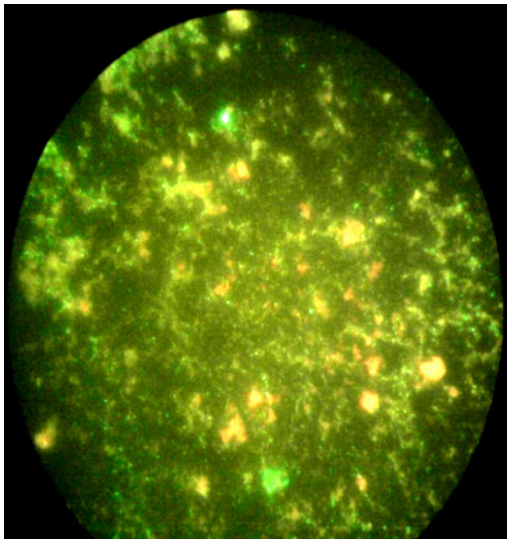
2b. Negative control for IgG (≤1:16)



2c. QF IFA Ph I IgG(1:256)



2d. QF IFA Ph II IgG (1:1024)



2a & 2b shows Positive control (1:32) and Negative control (1:16) for IgG;

2c & 2d shows test positive for dilution 1:256 for Ph I IgG and 1:1024 for Ph II IgG

Table –1: Comparison of QF Ph II IgM ELISA results with QF IFA IgM/IgG (Ph I/II)* (n=270)

Sl. No.	Combination of QF IFA	ELISA Positive** (n=150)	ELISA Negative# (n=120)	Total Positive (n=270)
1	Ph II IgM	2	-	2
2	Ph I and II IgM	1	-	1
3	Ph I IgG	3	3	6
4	Ph II IgG	8	0	8
5	Ph I and II IgG	12	8	20
6	Ph I + II IgM and Ph I IgG	4	-	4
7	Ph II IgM and Ph II IgG	5	-	5
8	Ph II IgM and Ph I + Ph II IgG	2	1	3
9	Ph II IgM and Ph I IgG	3	-	3
10	Both Ph I + II IgM and Ph I + II IgG	2	-	2
11	**Total (%)**	**42 (28.00%)**	**12 (10.00%)**	**54 (20.00%)** ##
12	All Negative (%)	108 (72.00%)	108 (90.00%)	216 (80.00%)

***** Significant titers for Ph I IgM/ Ph I IgG/Ph II IgM ≥ 1:16; For Ph II IgG ≥ 1: 256

****** ELISA positivity in one or more of the three commercial kits

ELISA negative in all three commercial kits

#Includes 7 Ph I IgM, 20 Ph II IgM, 38 Ph I IgG and 38 Ph II IgG

Table – 2: Results of QF IFA positivity among animal handlers/healthy controls (n=120)

Sl. No.	QF IFA IgM		QF IFA IgG		Occupation
	Ph I	Ph II	Ph I	Ph II	
1	Neg	Neg	1:256	1:128	Animal Attendants
2	Neg	Neg	1:128	1:512	Animal Attendants
3	Neg	Neg	1:128	1:512	Butcher
4	Neg	Neg	1:32	1:64	Butcher
5	Neg	Neg	1:64	1:256	Butcher
6	Neg	Neg	1:128	1:256	Butcher
7	Neg	Neg	1:128	1:512	Animal Attendants
8	Neg	Neg	1:128	1:256	Animal Attendants
9	Neg	Neg	1:64	1:512	Animal Attendants
10	Neg	Neg	1:256	1:128	Butcher
11	Neg	Neg	1:256	1:512	Butcher
12	Neg	1:32	1:64	1:256	Farmer
13-120*	Neg	Neg	Neg	Neg	

* 19 were Veterinarians/Animal attendants from veterinary clinics, 30 from butchers/slaughter house workers, 14 for farmers and 45 from healthy donors.

Analysis of 79 patients who provided both acute and convalescent samples

Out of 79 paired sera, 25 (31.64%) were positive for QF antibodies, including one patient who showed evidence of seroconversion – for Ph I + II IgM and IgG. Seven patients were positive for Ph I/II IgM/Ph I IgG in acute sera only, whereas 17 patients were positive for Ph I/II IgM and/or Ph I/ Ph II IgG in both acute as well as convalescent samples. The IFA titres ranged from 1:32 to 1:512 for IgM and 1:32 to 1:1024 for IgG (Ph I/II) antibodies. Remaining 54 paired samples were serologically negative for QF. In this study a four-fold increase in IFA titers was not observed in paired sera (Table – 3). Regarding the remaining 71 single, unpaired (acute) samples, 17 (23.94%) were positive for IFA IgM and/or IgG Ph I + II (Table – 4).

Table – 3: IFA Ph I/II IgM and Ph I/II IgG among paired samples with titres ≥1:32 (n=79)

Sl. No.	QF IFA IgM		QF IFA IgG		Animal contacts
	Ph I (A/C)	Ph II (A/C)	Ph I (A/C)	Ph II (A/C)	
1	Neg/Neg	Neg/Neg	128/64	256/128	
2	Neg/Neg	Neg/Neg	32/Neg	256/64	
3	128/Neg	128/Neg	1024/256	1024/256	
4	Neg/Neg	Neg/Neg	64/64	64/128	Sheep
5	Neg/Neg	Neg/Neg	Neg/Neg	512/64	Cow/goat
6	Neg/Neg	Neg/Neg	Neg/Neg	256/Neg	
7	Neg/Neg	512/Neg	Neg/Neg	512/128	
8	Neg/Neg	Neg/Neg	64/Neg	128/256	Sheep
9	Neg/Neg	128/512	Neg/Neg	256/512	
10	Neg/Neg	Neg/Neg	256/Neg	512/128	Goat
11	Neg/Neg	Neg/Neg	64/Neg	256/128	
12	Neg/Neg	Neg/32	128/64	512/256	Hen
13	Neg/Neg	Neg/Neg	64/64	256/512	
14	Neg/Neg	Neg/Neg	Neg/Neg	256/128	Cow/Cattle
15	Neg/Neg	Neg/Neg	Neg/Neg	256/128	
16	Neg/Neg	128/128	Neg/Neg	Neg/Neg	
17-18	Neg/Neg	64/128	64/128	256/512	Cat, Cow
19	Neg/Neg	Neg/Neg	512/256	512/256	

Sl. No.	QF IFA IgM		QF IFA IgG		Animal contacts Ph II (A/C)
	Ph I (A/C)	Ph II (A/C)	Ph I (A/C)	Ph II (A/C)	
20	Neg/Neg	Neg/Neg	Neg/Neg	256/256	Abattoir
21	Neg/Neg	Neg/Neg	128/Neg	256/Neg	
22	Neg/Neg	Neg/Neg	64/64	256/256	
23	Neg/Neg	64/Neg	Neg/Neg	Neg/Neg	Abattoir
24	64/Neg	128/Neg	Neg/Neg	Neg/64	
25	Neg/32	Neg/32	Neg/32	Neg/128	Dog
26-79	Neg/Neg	Neg/Neg	Neg/Neg	Neg/Neg	Hen, Cow[#]

Eight patients had animal contact

A – Acute; C - Convalescent

Table – 4: Results of QF IFA positivity among patients who provided only acute samples (n=71)

Sl. No.	QF IFA IgM		QF IFA IgG		Animal contacts
	Ph I (A/C)	Ph II (A/C)	Ph I (A/C)	Ph II (A/C)	
1	1:64	1:128	1:64	Neg	
2	Neg	1:64	Neg	1:512	
3	1:128	1:128	1:64	Neg	
4	Neg	Neg	1:128	1:512	
5	Neg	Neg	1:64	1:256	
6	Neg	1:128	Neg	1:512	
7	Neg	Neg	1:128	1:512	
8	Neg	Neg	1:256	1:512	
9	Neg	1:128	1:32	Neg	
10	1:64	1:32	1:64	Neg	
11-12	Neg	1:64	1:64	Neg	
13-14	Neg	Neg	Neg	1:256	
15-17	Neg	Neg	1:128	1:256	
18-71	Neg	Neg	Neg	Neg	Cow (4),Sheep (4), Dog (1), Hen (1)

A – Acute; C - Convalescent

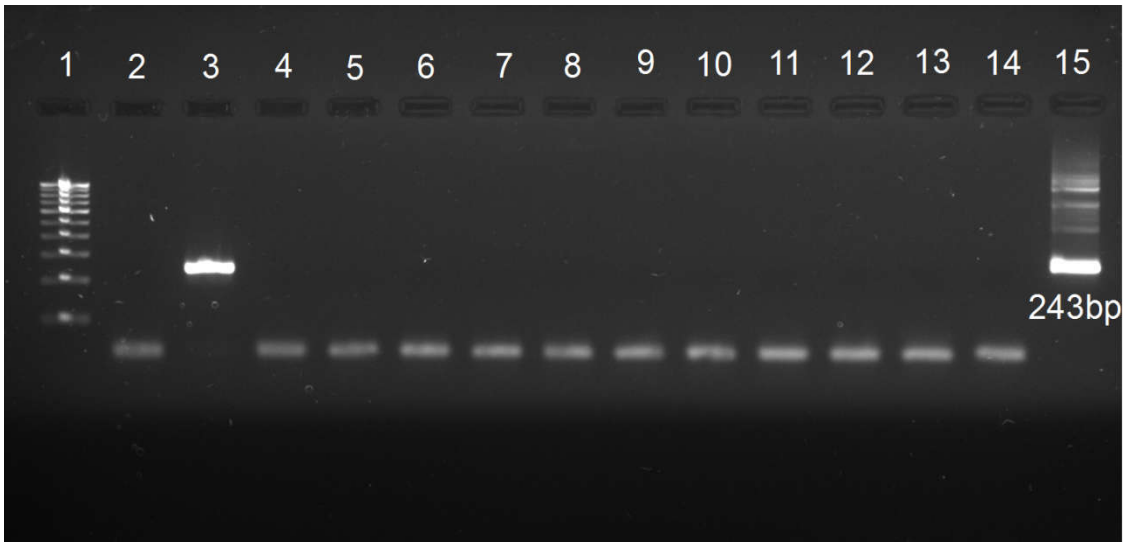
6.2 Molecular Diagnosis of Q fever

Three different tests were performed for the molecular diagnosis of Q fever viz., Nested PCR (N-PCR), Real Time PCR (qPCR) (In-house as well as commercial q16 qPCR) and LAMP.

6.2.1. Nested PCR (N-PCR)

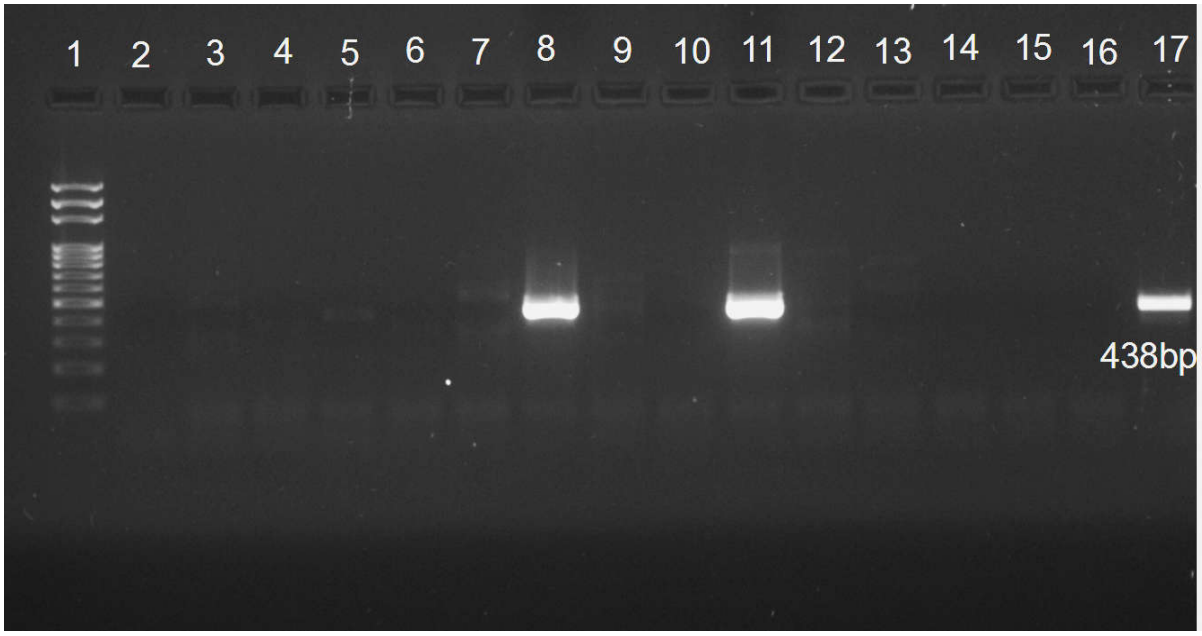
N-PCR targeting IS1111 gene identified 22 samples (8.15%), which showed band size at 243bp (Figure – 3). The second target of COM1 had a positivity of 5.55% (15/270) and its amplicon size was 438bp (Figure – 4). Only one sample was positive for both the genes. The overall positivity of N-PCR for IS1111 and/or COM1 is 13.33% (36/270).

Figure – 3: Nested PCR for IS1111 gene of *Coxiella burnetii*



Lane 1 – 100bp DNA Ladder; Lane 2 – Negative control; Lane 3 – Test positive, Lane 4-14 – Test negative and Lane 15 –Positive control band at 243bp

Figure – 4: Nested PCR for COM1 gene of *Coxiella burnetii*

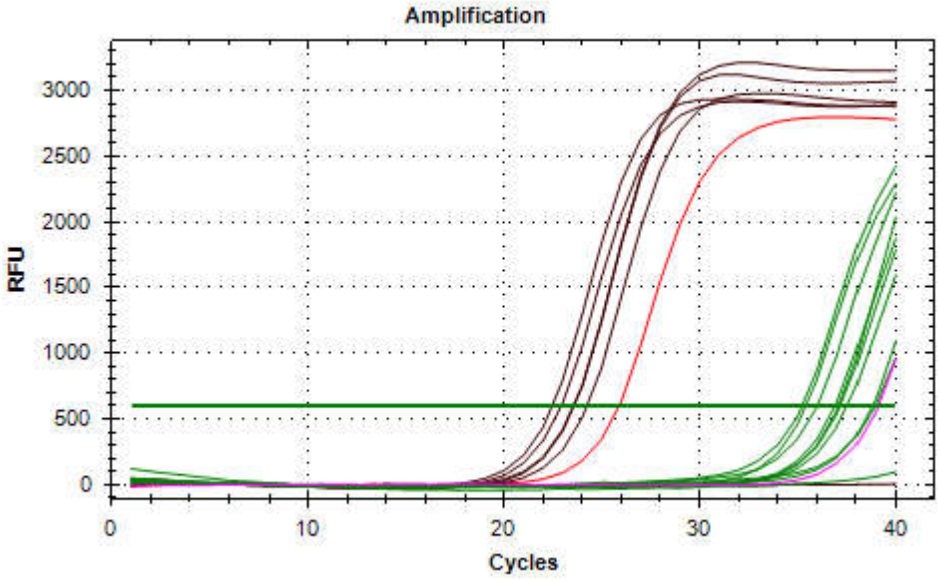


Lane 1 – 100bp DNA Ladder; Lane 2 – Negative control; Lane 3-7, 9-10 & 12-16 – Test negative and Lane 8 & 11 – Test positive; Lane 15 –Positive control band at 438bp

6.2.2. In-house Real Time PCR (qPCR)

qPCR positivity for IS1111 gene was 8.15% (22/270) and 6.30% (17/270) for COM1 gene. Four samples were positive for both genes. Ct values for qPCR positive samples varied from 26.49 – 34.86 in IS1111 as well as COM1 gene (Figure – 5). Overall positive in our in-house qPCR was 12.96% (35/270). Positive samples were confirmed in 2.0% agarose gel electrophoresis stained with Ethidium bromide.

Figure –5: Amplification of *Coxiella burnetii* DNA in qPCR (In-house)



Red – Known positive control; Brown –Internal control (Beta actin); Green – Patients' samples; Pink – Negative control

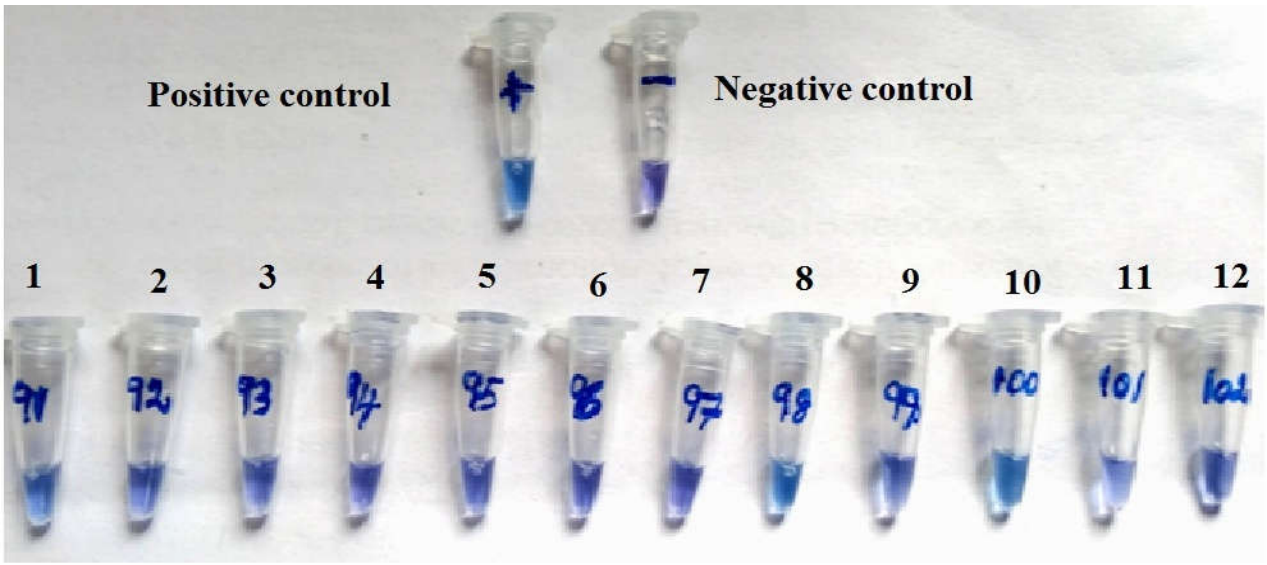
6.2.3. Validation of QF Commercial Real Time PCR (Gen-sig, Primer Design Pvt. Ltd, United Kingdom):

A preliminary report identified just two samples out of 72 patients and 24 healthy donors were tested (2.78%) by this commercial kit which targets 97kDa gyrA gene (DNA gyrase) (Pradeep et al., 2017). Later, the addition of more samples (n=270) picked up eight positive cases (2.96%) by this qPCR. However, there is not much of difference in the positivity, even with the increase in sample size.

6.2.4. Loop Mediated Isothermal Amplification:

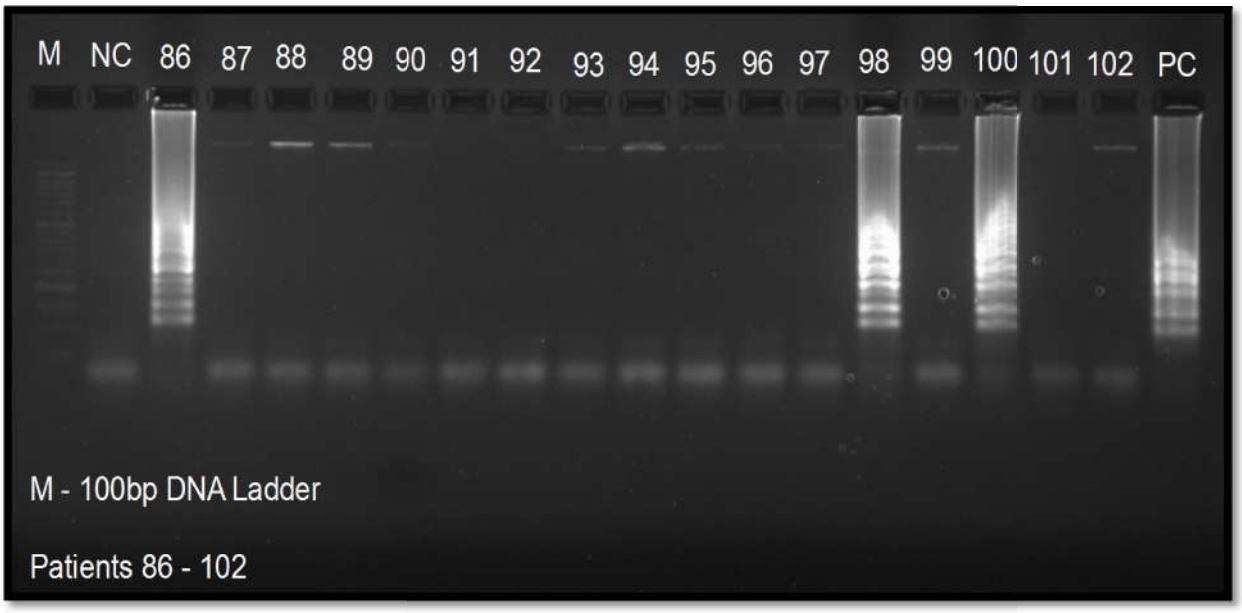
Positivity of the LAMP for 270 samples was 28 (10.37%) and 19 (7.03%) for IS1111 and COM1 genes respectively. Four samples were positive for both the genes. Overall positivity of LAMP for IS1111 and/or COM1 gene was 15.93%. Results of LAMP was interpreted by visual detection of color change from dark blue to sky blue (Figure – 6) and further confirmed by 2.0% agarose gel electrophoresis with Ethidium bromide dye (Figure - 7). For confirmation of LAMP products, one representative positive sample was selected for each gene and PCR was performed using outer primers (F3 & B3) and amplified products were sequenced and these sequences were analyzed by NCBI database and QF was confirmed.

Figure – 6: Image of visual detection of LAMP products



Upper row - positive and negative controls; Lower row – Tube 1-7, 9, 11&12 – Test negative and Tube 8 & 10 – Test positive

Figure – 7: Images of 2.0% agarose gel analysis of LAMP products



M – 100bp DNA Ladder; NC – Negative control; PC - Positive control; Samples 86, 98 and 100 – Test positive and Samples 87-97, 99 and 101-102 – Test negative

6.3. Results of different combination of three molecular tests for IS1111 and COM1 gene

The results of N-PCR, qPCR and LAMP with different combinations are shown in Table – 5. On the whole, molecular evidence of QF positivity was observed among IS1111 gene is about 27 (10.00%) and for COM1 gene 20 (7.40%).

Table - 5: Comparison of N-PCR, In-house qPCR and LAMP assays for IS1111 and COM1 genes (n=270)

Categories of positivity	Positive for IS1111 Gene only	Positive for COM1 Gene only	Positive for both IS1111 and COM1	Total Positive
N- PCR Only	00	01	00	01
qPCR only	00	00	00	00
LAMP only	00	00	00	00
N-PCR + qPCR	00	00	00	00
N-PCR + LAMP	05	02	00	07
qPCR + LAMP	05	02	00	07
N-PCR + qPCR + LAMP	13	11	04	28
Total (%)	**23 (8.52%)**	**16 (5.92%)**	**04 (1.48%)**	**43 (15.92%)**

Note:

IS1111 gene

Nested PCR Positivity → 22; Real Time PCR positivity → 22; LAMP positivity → 27

COM1 gene

Nested PCR Positivity → 15; Real Time PCR positivity → 17; LAMP positivity → 19

6.5. Clinical and Laboratory Findings

Clinical and laboratory findings of QF positive and negative febrile patients are presented in Table - 6. Among QF positive and negative patients, only four parameters were of statistical significance viz., chills and rigour (p=0.0327), rash (p=0.0274), hepato-splenomegaly (p=0.0324**)** and increased liver enzymes (p=0.0194).

Table – 6: Clinical and laboratory findings among febrile patients (n=150)

Clinical/Laboratory findings	QF Positive patients (n=56)	QF negative patients (n=94)	Total (%)	p value*
Fever (≤14 days)	48	84	132 (88.00%)	0.6853
Fever (≥14 days)	8	10	18 (12.00%)	0.6853
Headache	27	44	71 (47.33%)	0.8675
Chills & Rigor	46	62	108 (72.00%)	**0.0327**
Rash	1	5	6 (4.00%)	**0.0274**
Lymphadenopathy	1	3	4 (2.67%)	0.6052
Myalgia	17	22	39 (26.00%)	0.3477
Eye pain	1	3	4 (2.67%)	0.6052
Cough & Expectoration	25	38	63 (42.00%)	0.6127
Abdominal Pain	26	47	73 (48.67%)	0.6721
Altered Sensorium	3	1	4 (2.67%)	0.1467
Bleeding episodes	2	2	4 (2.67%)	0.6295
Hepto-splenomegaly	9	30	39 (26.00%)	**0.0324**
Malaise	4	3	7 (4.67%)	0.4252

Nausea	11	24	35 (23.33%)	0.4095
Vomiting	19	40	59 (39.33%)	0.2956
Pneumonitis	15	19	34 (22.67%)	0.3524
Neck Rigidity	1	2	3 (2.00%)	0.8850
Joint Pain	3	8	11 (7.33%)	0.5385
Chest Pain	3	7	10 (6.67%)	0.7443
Laboratory Findings				
Increased Liver enzymes (AST/ALP/AP)*	28	29	57 (38.00%)	**0.0194**
Low Platelet count (<1.5 Lakhs/cumm)	16	32	48 (32.00%)	0.4872
Creatinine (>1.0 mg/dL)	12	11	23 (15.33%)	0.1098
Other tests positive[#]	10	13	23 (15.33%)	.50787

* P values <0.05 were statistically not significant

- Co-infection with other diseases like Typhoid (11), Dengue (10), Pl. vivax (02)

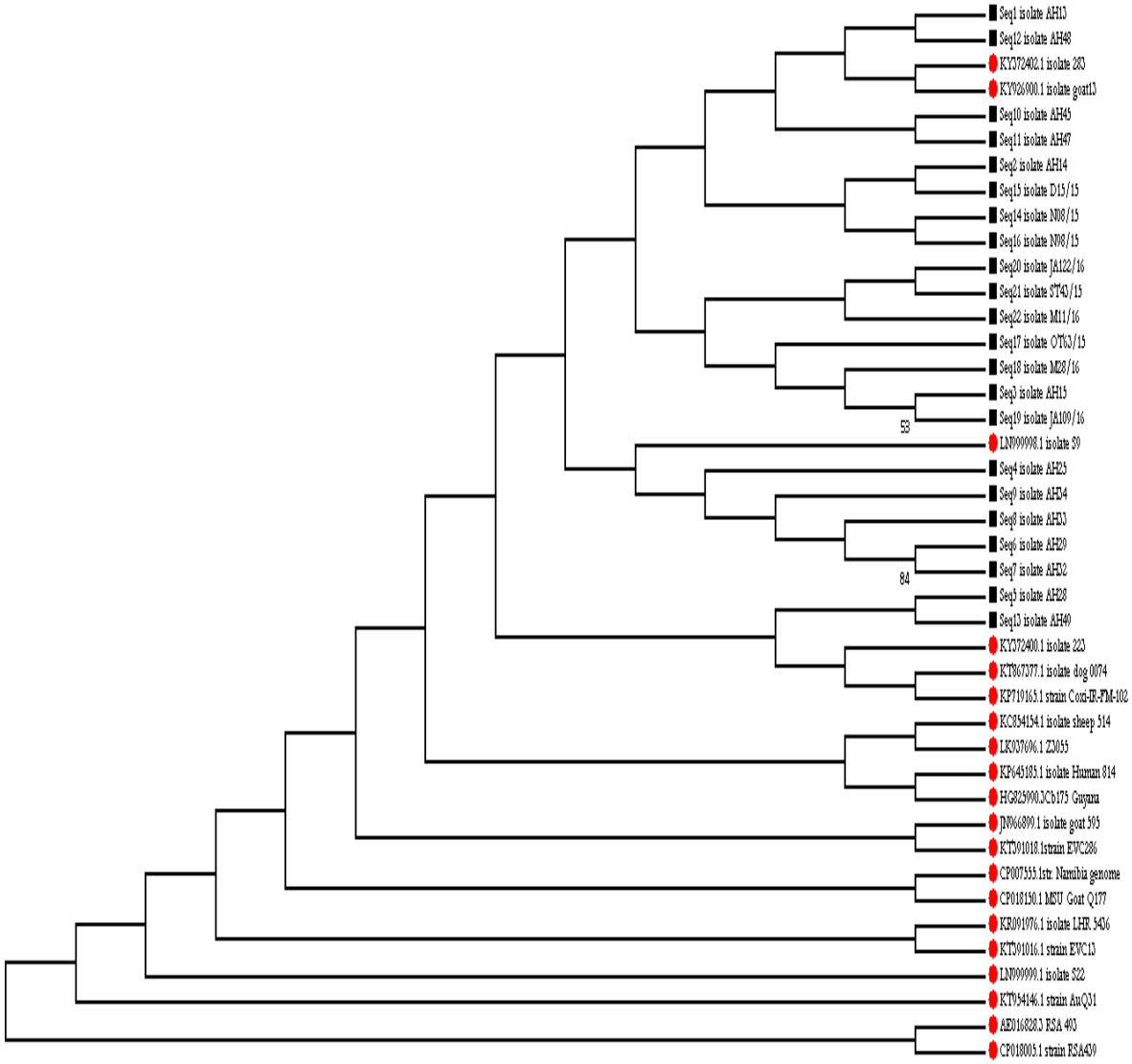
6.6. <u>Gene sequencing:</u>

The specificity of the 23 sequences was determined by using BLAST (Basic Local Alignment Search Tool) analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) which showed 99-

100% maximum identity with the other isolates in the NCBI database (AH13, AH48, AH14, AH43, AH47, AH25, AH45, D15/16, N08/15 and N78/15). These sequences were then aligned using biological sequence alignment editor software MultAlin (Corpet, 1988). Twenty three nucleotides were aligned with corresponding sequences, which were retrieved from GenBank by using ClustalW (https://www.ebi.ac.uk/Tools/msa/clustalo/) and phylogenetic tree was constructed for the two genes (Figure 8 & 9) using MEGA software Version 10.0 (Kumar et al., 2018).

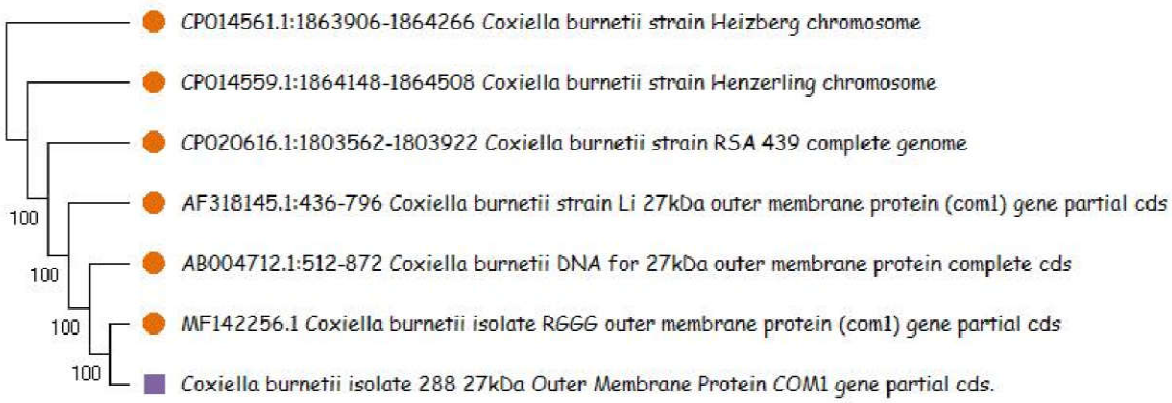
The aligned sequences were submitted to GenBank and obtained accession numbers MG548608 to MG548629 for IS1111 were published and MH758791 for COM1 gene (yet to be published) in public domain. Phylogenetic analysis was made in IS1111 gene and shows that the study sequences 5 and 13 were weakly grouped along with the reference strains (KY372400.1, KT867377.1 and KP719165.1) forming a separate lineage. The study sequences 3,4,6,7 and 9.forms a cluster along with the reference strain LN999998.1 obtained from Bangalore. Within the clusters the isolates 6 and 7 forms a clade and supported by a bootstrap value of 84%. Remaining study sequences forms a large cluster along with reference strains from the regions of Colombia (KY372402.1) and Brazil (KY926900.1). The study sequences are more similar to each other forming a subclade. It forms a clade along with the reference sequences KY372402.1and KY926900.1. The study isolates 3 and 19 share common ancestor which supported by a bootstrap value of 53%. The phylogenetic analysis of COM1 gene, the study sequence MH758791 showed 99% maximum identity with the Indian strain MF142256.1 from Uttar Pradesh. The other reference isolates AF318145.1, AB004712.1, CP014561.1 CP014559.1 and CP020616.1 forms a separate lineage from the study isolate. This may indicates that few study isolates showed a more resemblance with our Indian isolates, when compared to other reference strains

Figure – 8: Phylogenetic analysis of *Coxiella burnetii* on Transposase IS1111 gene by Maximum Likelihood method



Green color indicates study sequences and Red indicates Reference sequences of *C. burnetii*

Figure – 9: Phylogenetic analysis of *Coxiella burnetii* on COM1 gene by Maximum Likelihood method


CP014561.1:1863906-1864266 Coxiella burnetii strain Heizberg chromosome
CP014559.1:1864148-1864508 Coxiella burnetii strain Henzerling chromosome
CP020616.1:1803562-1803922 Coxiella burnetii strain RSA 439 complete genome
AF318145.1:436-796 Coxiella burnetii strain Li 27kDa outer membrane protein (com1) gene partial cds
AB004712.1:512-872 Coxiella burnetii DNA for 27kDa outer membrane protein complete cds
MF142256.1 Coxiella burnetii isolate RGGG outer membrane protein (com1) gene partial cds
Coxiella burnetii isolate 288 27kDa Outer Membrane Protein COM1 gene partial cds.
100
100
100
100

Orange color shows reference strains and violet indicates study isolates

7. DISCUSSION

7.1. Q fever diagnostic algorithm by Jager et al., 2011

According to Jager et al., 2011, the diagnostic algorithm for acute Q fever is the presence of both IgM Ph II and IgG Ph II. IgG Ph II with or without IgG Ph I antibodies is considered as a past resolved Q fever. Any other combinations of antibodies are to be subjected to PCR within the 1-14 days of febrile illness.

7.2 Q fever diagnostic algorithm by Anderson et al., 2013 (CDC, Atlanta)

CDC criteria for the diagnosis of acute as well as chronic Q fever was proposed by Anderson et al., 2013 are presented below. It is to be noted that the difference between the two algorithms is only reference to results in serological tests. Positivity in a molecular test is considered as confirmatory by both groups.

Acute Q fever:

Laboratory supportive with case classification (one or more of the following):

Single titer of ≥1:128 to Ph II IgG by IFA or Elevated QF phase II IgG or IgM antibody by ELISA, dot-ELISA, or latex agglutination

Probable acute Q fever: Clinical evidence of infection with laboratory-supportive results

Laboratory Confirmed with case definition (one or more of the following):

Fourfold rise in Ph II IgG antibody titer by IFA in paired sera. Demonstration and isolation of *C. burnetii* in a clinical specimen by PCR/IHC and culture.

Confirmed acute Q fever: Laboratory-confirmation with clinical evidence of infection or an epidemiological link to a laboratory-confirmed case

Chronic Q fever: (one or more of the following):

Laboratory supportive: Single titre of IgG Ph I antibodies between ≥1:128 and <1:800/1:1024 to *C. burnetii.*

Probable chronic Q fever: Clinical evidence of infection with laboratory-supportive results

Laboratory confirmed (one or more of the following):

C. burnetii IgG Ph I titer of ≥1:800/1:1024 by IFA. Detection and isolation of *C. burnetii* in a clinical specimen by PCR/IHC/Culture.

Confirmed chronic Q fever: Clinical evidence of infection with laboratory confirmation

7.3. Role of Ph II IgM ELISA in the diagnosis of acute Q fever

During the past two decades, specific serological tests like ELISA and IFA are employed for the serodiagnosis of QF (Vaidya et al., 2008; Schneeberger et al., 2010; Jager et al., 2011; Herrmann's et al., 2013; Stephen et al., 2017). While, Ph II IgM ELISA commercial kits like Serion/Virion (Jager et al., 2011), Vircell (Kantsø et al., 2012) and NovaTec (Szymanska-Czerwinska et al., 2015) have performed well in overseas countries, a preliminary study has exposed the unreliability of these kits which are imported and made available in India and hence the 'gold standard' IFA has become mandatory for QF diagnosis (Stephen et al., 2017). Jager et al., 2011 utilized QF Ph II IgM ELISA Serion/Virion kit for initial screening of the samples followed by confirmation with IFA and/or PCR. According to Raven et al., 2012, QF Ph II IgM ELISA positivity as a standalone finding cannot be considered for confirming acute Q fever.

Table-1 compares IFA results with Ph II IgM ELISA for 270 archived samples (150 ELISA positive and 120 ELISA negative samples). Fifty four samples were positive (20.00%) in QF IFA IgM and/or IgG (Ph I and/or II) in titres considered as significant by the IFA commercial kit. Solitary Ph II IgM positivity was found in two patients with titres of 1:64 and 1:128 respectively and may be considered as acute Q fever. However, Raven et al., 2012 do not consider this as pointing to acute Q fever. On the basis of our serological results (positivity in Ph I/II/IgM/IgG) and significant titres according to Fuller labs IFA kits' brochure, it could be inferred that 51 belong to acute Q fever (19.25%) (Recent or active infection) with presence of

Ph I/II IgM and Ph II IgG, three (1.11%) could be considered as chronic Q fever due to IgG Ph I positivity.

7.4. Interpretation of serological results as per algorithm by Jager et al., 2011

Regarding 79 patients who provided both acute and convalescent blood samples (Table-3), six (7.59%) were diagnosed as acute Q fever with both Ph II IgM and Ph II IgG positivity, fourteen (17.72%) as past resolved Q fever, (Ph II IgG positivity with or without Ph I IgG) and five as inconclusive (6.33%) (Only Ph II IgM/ only Ph I IgG or Ph I+II IgM) as per the algorithm of Jager et al., 2011. Remaining 54 paired samples were negative for all four IFA tests (IgM Ph I+II and IgG Ph I + II). With reference to 71 unpaired samples (Table – 4), two patients (2.81%) belongs to acute QF category, nine (12.67%) may be considered as past resolved QF and six (8.45%) as inconclusive. Remaining 54 acute samples were seronegative for QF.

Table - 2 analyses serological evidence of QF among animal handlers and healthy controls (voluntary blood donors). Only one farmer (1.33%) had acute QF positivity with presence of Ph II IgM, Ph II IgG and Ph I IgG antibodies. Animal attendants and butchers (four each) (5.33%) were considered belong to past resolved QF. Three had inconclusive results (4.00%), an animal attendant and two butchers. No seropositivity was observed among the remaining 63 animal handlers and all 45 blood donors.

7.5. Interpretation of serological results as per algorithm by Anderson et al., 2013

According to CDC criteria, based on serology, 37 out of 270 samples (13.70%) were considered as a laboratory supportive acute Q fever with significant titre of ≥1:256 for IgG Ph II and two cases (0.74%) were found to be in the category of laboratory supportive chronic Q fever with Ph I IgG titres of ≥1:256 to ≤1:1024. Only one patient could be considered as belonging to laboratory confirmed chronic Q fever with titre of 1:1024 for Ph I IgG antibodies.

7.6. Comparison of three molecular tests targeting both IS1111 and COM1 genes

Early detection of *C. burnetii* DNA is achieved by application of the sensitive and specific N-PCR, qPCR and LAMP. There are two gene targets - the most conserved regions of chromosomal genes IS1111 with detection limit of 21 copies and COM1 gene, a single copy in the genome (de Bruin et al. 2013). In the current study, all 270 samples were subjected to the three molecular tests targeting both genes. Table – 5 compares the results of N-PCR, In-house qPCR and LAMP assays for IS1111 and COM1 genes. Overall positivity in any one or more molecular tests was 43 (15.92%), comprising 23 for IS1111 (8.52%), 16 for COM1 gene (5.92%) and four (1.48%) positive for both the genes. Regarding the positivity of the three molecular tests to detect IS1111/COM1 targets, LAMP has more in number (17.41%) followed by N-PCR (13.33%) and qPCR (12.97%). Pan et al., 2013, reported QF LAMP positivity of 33.33% compared to qPCR positivity of only 8.25%. To our knowledge, so far there has been no other report of QF LAMP on febrile patients/animal handlers in the world literature. Nested PCR is useful for direct identification of QF in clinical samples (Zhang et al., 1998). *C. burnetii* DNA is detectable in the blood during the early phase of the infection, approximately between 0-20 days only, and it disappears from the blood immediately after the appearance of antibodies (Schneeberger et al., 2010).

7.7. Comparison of QF results with two algorithms from Netherlands (Jager et al., 2011) and USA (Anderson et al., 2013)

Our findings on QF positivity (n=85) is compared against the background of two different algorithms based on serology and molecular tests.

Comparison with algorithm of Jager et al., 2011:

Twenty nine patients out of 150 ELISA positive patients (19.33%) could be considered as having acute Q fever due to the presence of both IgM +IgG Ph II antibodies and/or positivity in molecular tests.

Sixteen patients (10.67%) come under the category of past resolved Q fever on the basis of positivity in Ph II IgG and/or Ph I IgG IFA and absence of *C. burnetii* DNA in the molecular tests.

The third category of eleven patients is considered as "inconclusive": Among them, two had Ph II IgM alone, three had both Ph II IgM and Ph I IgG antibodies. Five were positive for Ph I IgG only and one patient was positive for Ph I+II IgM and Ph I IgG.

Out of 120 ELISA negative samples, seven animal handlers (6.67%) were considered as past resolved QF on serological findings and 21 (17.50%) as laboratory confirmed acute Q fever due to the detection of *C. burnetii* DNA. All 91 samples were negative for QF serology as well as molecular tests.

Comparison with algorithm of Anderson et al., 2013 (CDC)

In the present study, among 150 patients, 21 had probable acute Q fever (14.00%) and 23 with confirmed acute Q fever (15.33%). Out of 75 animal handlers, seven (9.33%) showed evidence of laboratory supportive and 21 (28.00%) laboratory confirmed acute Q fever. One patient had IgG Ph I titre of 1:1024 and considered as laboratory confirmed chronic Q fever. Two animal handlers had Ph I IgG in titre of 1:256 can be considered as belonging to laboratory supportive chronic QF. None of the 45 voluntary blood donors were positive for either serology or molecular tests

In the present study the evidence of QF positivity by serology and/or molecular diagnosis in febrile patients was 37.33%. So this positivity is quite high when compared to a recent study

from Karnataka (4.5%) (Kumar et al., 2017). However these authors carried out qPCR for IS1111 gene only.

Vaidya et al., 2008 reported QF positivity in Uttar Pradesh about 25.68% by isolation, IFA and qPCR targeting IS1111 gene among women with abortions. Various case reports of acute Q fever pneumonia (Panjwani et al., 2015) and neonatal sepsis (Arthi et al., 2013) are available in the recent Indian literature.

In the present study, against the gold standard IFA, statistical significance of p=0.0453, p=0.0105 and p=0.0281 were recorded in N-PCR, qPCR and LAMP respectively for the presence of IS1111 gene. On the other hand, keeping IFA as reference, no statistical significance was observed in all three PCR targeting COM1 gene. A comparison of the three molecular tests viz., N-PCR, qPCR and LAMP revealed that there is no statistical significance among these tests for IS1111 (p=0.5776) and COM1 gene (p=0.7779).

To our knowledge, only one study has been conducted in India using this COM1 gene and that too in avian coxiellosis but not in human Q fever (Das et al 2013).

7.8. Comparison between commercial qPCR kit and In-house qPCR:

Commercial Portable q16 qPCR was performed and validated for the first time in India, which targets 97kDa gyrA gene (DNA gyrase). It yielded a low positivity of 2.96%, when compared with our in-house qPCR, which showed positivity of 12.96%. This perhaps could be explained due to specific primer binding sites. Among the eight patients who were positive in commercial qPCR kit (DNA gyrase gene), four were positive for IS1111 gene and one for COM1 gene in our in-house qPCR. Remaining three was positive only in commercial qPCR. Regarding the serological results of these eight patients were positive in commercial qPCR, comprising of two had both IgM Ph II and IgG Ph II and could be categorized as laboratory confirmed acute Q fever by both algorithms. One patient was positive for IgG Ph II only and

may be considered as past resolved Q fever according to Jager et al., and laboratory supportive acute Q fever as per CDC Criteria. However, on the basis of detection of *C. burnetii* DNA in the commercial kit, all the eight patients are to be categorized as acute Q fever by both algorithms. Advantages of this kit are two-step process, easy to handle, does not require much space and electrical power like other Real Time PCR instruments. Sensitivity wise this commercial qPCR is inferior to our in-house qPCR and also is expensive and need to be imported.

7.9. Loop Mediated Isothermal Amplification

LAMP is relatively fast, cost-effective test when compared to qPCR (Pan et al., 2013). Both are highly sensitive and specific for diagnosis of QF. In the present study, LAMP appears to be more sensitive than N-PCR and qPCR. LAMP primers for IS1111 gene were self designed by using Primer Explorer software Version 4.0. The outer primers were confirmed by gene sequencing. The primers used for COM1 gene were those used in Italy by Raele et al., 2015. The major drawback of LAMP is the need for optimizing $MgSO_4$ concentration of the reaction mixture on a day-to-day basis so as to obtain the desired colour change in the positive and negative samples. To our knowledge, inclusion of COM1 gene for detection of *C. burnetii* DNA in febrile patients as well as animal handlers using LAMP is the first report in the Asian Continent.

7.10. Clinical and laboratory parameters

Clinical and laboratory parameters of QF positive and negative febrile patients are presented in Table – 5. The parameters with statistical difference were: Chills and rigour ($p=0.0327$), rash ($p=0.0274$), hepato-splenomegaly ($p=0.0324$**)** and increased liver enzymes ($p=0.0194$). Ten QF positive patients had co-infection with other diseases like Typhoid and Dengue

7.11. Serological false positivity in Q fever

IgM positivity alone is not considered as a proof of acute Q fever by different researchers (Raven et al., 2012; Anderson et al., 2013). This is because QF IgM may be falsely positive in different diseases including spotted fever group and it persists in blood upto one year (Anderson et al., 2013). According to Kantso et al., 2009, cross-reactivity with other spotted fever or typhus group rickettsiae may be seen due to phylogenetic relationship between the two species.

7.12. Phylogenetic analysis

Phylogenetic tree was constructed by Maximum Likelihood methods based on IS1111 and COM1 sequence of *C. burnetii*. Majority of our study sequences are highly similar with Indian reference strains from Bangalore and Uttar Pradesh compared to the other reference isolates.

7.13. Cost analysis of serological/molecular tests:

For IgM/IgG IFA, the cost is about Rs. 1500 /- per each test. Molecular tests per sample cost about Rs. 800 for N-PCR, Rs. 1000/- for qPCR and Rs. 500/- for LAMP for each gene target. Real Time PCR instrument is very expensive compared to that of gel PCR and LAMP

8. CONCLUSION

Due to poor sensitivity and specificity of three commercial QF Ph II IgM ELISA kits available in India, the inclusion of IFA has become mandatory. Selection of Primers is critical for PCR work and it is helpful in the early diagnosis of QF while antibodies may appear later. On the whole, IS1111 transposon is a better target than COM1 gene for detecting *C. burnetii* DNA in clinical samples. COM1 gene target is quite helpful for identifying additional cases of QF in symptomatic as well as asymptomatic patients. Only few studies in the world literature incorporated COM1 gene. In the present study, the sensitivity of N-PCR and qPCR are the same

for both the genes. But, the concordance was more in qPCR than N-PCR for both the gene targets.

LAMP is a platform for the molecular detection of important zoonotic infections in developing countries. It is superior to qPCR and N-PCR due to its ease of operation. The setback of LAMP is optimizing $MgSO_4$ concentration of the reaction mixture daily.

Our findings suggest that out of 150 febrile patients, 14.00% had probable acute Q fever and 15.33% with confirmed acute Q fever by CDC criteria. But by algorithm of Jager et al, 19.33% had acute Q fever and 10.67% come under the category of past resolved Q fever. Clinical features such as Rash, Chills and rigour, hepato-splenomegaly and increased transaminase levels showed statistical significance among the febrile patients.

Among animal handlers, more number of butchers had active infection for this zoonotic disease. This may be due to repeated exposure to *C. burnetii* infected animals. However, the animal handlers as well as slaughter house workers should be made aware of the risks and control measures to be adopted. Therefore, a large number of studies covering a larger population is needed to validate the N-PCR, qPCR and LAMP assays. The present study found that there was no statistical difference among the three molecular tests for QF diagnosis. In conclusion it may be said that a combination of N-PCR, qPCR and LAMP is helpful for identifying more number of cases. When compared to PCR assays, LAMP is cheap, rapid, less time-consuming, highly sensitive and does not require any costly instruments. Commercial portable q16 qPCR is a two step process, more sensitive and specific, detects <5 copies/µl of the targets. It can be even setup at resource poor settings. However this kit is less sensitive than in-house qPCR.

Our study sequences are highly similar (99-100%) to other reference sequences which are available in the NCBI database. Phylogenetic analysis shows that only a few study isolates are

strongly grouped with reference isolates particularly to our Indian strains. Majority of our isolates are weakly grouped with other reference isolates in the GenBank. Finally, the present study shows the true picture of *C. burnetii* infection among febrile patients and animal handlers of Puducherry and neighbouring state of Tamil Nadu, based on serological and/or molecular tests.

9. LIMITATION OF THE STUDY

Among the 150 patients included in the study, paired samples were available only for 79 patients.

Due to cost constraints, only half of the PCR positive samples were sequenced.

Follow-up of animal handlers could not be done, which could be taken up for future research work.

10. SUMMARY

1. QF seropositivity of 20.00% was observed in Ph I and/or Ph II IgM and/or IgG in IFA among 270 participants residing in Puducherry & neighbouring Tamil Nadu.

2. Among 150 QF IgM Ph II ELISA positive patients, 42 could be confirmed by IFA (28.00%). However, only 23 patients (15.33%) had prior exposure to *C. burnetii* as per DNA detection in the Molecular test(s). Among 120 QF IgM Ph II ELISA negative samples, 12 could be confirmed by IFA (10.00%).

3. Out of 75 animal handlers, QF positivity was quite high among butchers/slaughter house workers (52.77%) followed by Veterinarians/Animal attendants (33.33%) and farmers (13.33%) by using serological and/or molecular evidence of *C. burnetii*.

4. QF IFA kits (Fuller Lab, California, USA) has been successfully validated for the first time in India and was found satisfactory. However it is recommended that these IFA kits may be further validated using more number of samples.

5. All 45 voluntary blood donors were negative for *C. burnetii* antibodies as well as *C. burnetii* DNA.

6. LAMP for Q fever diagnosis targeting IS1111 as well as COM1 gene is being performed for the first time in India and only few reports are available in the world literature.

7. LAMP primers for IS1111 gene were successfully designed from the isolate (KT391019.1) and utilized in this research work.

8. There was no statistical significance was observed among the three different molecular tests for QF diagnosis

8. The commercial qPCR (Gen-sig, UK) has a poor sensitivity of 2.96% only.

9. GenBank Accession Numbers for 22 sequences for IS1111 were published in NCBI database (MG548608-MG548629). One sequence for COM1 was deposited with NCBI and accession number (MH758791) was obtained (yet to be published). These sequences had 99-100% maximum identity with other reference isolates from GenBank records.

10. Phylogenetic tree has revealed that the study sequences are more similar with reference strains from India, Colombia and Brazil.

11. Q fever serological as well as molecular diagnosis is quite expensive and efforts should be made to manufacture them in India, preferably incorporating local isolates.

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12. LIST OF PUBLICATIONS FROM Ph.D WORK.

1. Pradeep J, Stephen S, Ambroise S, Gunasekaran D. Diagnosis of acute Q fever in Puducherry: Detection of Coxiella burnetii DNA by Real Time PCR, employing a commercial Genesig easy kit. **J Clin Diag. Res**. 2017;11(9):DC10-18.
2. Stephen S, Ambroise S, Pradeep J, Gunasekaran D, Sangeetha B, Sarangapani K. Unreliability of three commercial Coxiella burnetii phase II IgM ELISA kits for the seroscreening of acute Q fever in human cases. **Indian J Med Res.** 2017;146:386-391
3. Pradeep J, Kumar S, Stephen S, Kamboj DV, Gunasekaran D, Hanifah M. Prevalence of acute Q fever in humans in Puducherry and neighbouring Tamil Nadu by indirect immunofluoresence and real time PCR. **Indian J Med Res.** (Accepted for Publication-2018)

12. 1. Publications on Coxiellosis in livestock

1. Pradeep J, Stephen S, Sangeetha B, Antony PX. Seroprevalence of Bovine Coxiellosis in Puducherry and Tamil Nadu – with a note on its relevance to human Q fever. **Biomedicine**: 2016; 36(3): 51-54.
2. Pradeep J, Stephen S, Pooja P, Akshayavardhini A, Sangeetha B, Antony PX (2017) Coxiellosis in domestic livestock of Puducherry and Tamil Nadu: Detection of Coxiella burnetii DNA by polymerase chain reaction in slaughtered ruminants, **Veterinary World**, 10(6): 667-671.

13. LIST OF CONFERENCES ATTENDED (NATIONAL AND INTERNATIONAL)

1. Participated and made an **oral presentation** in the National conference Sponsored by UGC on "Emerging Infectious Diseases and Curative Measures" organized by PG Department of Microbiology, Kamarajar College, Thoothukudi on 22nd & 23rd December, 2016.
2. Participated and made **an poster presentation** in the International conference on "Microbiome in Health, Disease and Environment" organized by Central Inter-Disciplinary Research Facility (CIDRF) on 3rd & 4th March, 2017.
3. Participated and made an **poster presentation** in the national conference on "Environment, Health and Disease Theme: Ecogenetics & Toxicogenomics" organized by Central Inter-Disciplinary Research Facility (CIDRF) on 12th February, 2018.